The Anti-Malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction

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	Please find attached the manuscript with the suggested final changes. Kind regards, Christoph Thiemermann

The Anti-malarial Drug Artesunate Attenuates Cardiac Injury in a Rodent Model of Myocardial Infarction

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ABSTRACT

Ischaemic heart disease remains the leading cause of morbidity and mortality in the Western world. Artesunate is the WHO-recommended drug of choice for complicated malaria (with organ failure). The administration of high doses of artesunate is safe in healthy volunteers (up to 8 mg/kg i.v) and patients with severe malaria (2.4 mg/kg i.v). We investigated the effects of artesunate (1 mg/kg) or its active metabolite dihydroartemisinin (DHA; 0.1 mg/kg) in a model of transient myocardial ischaemia/reperfusion (I/R) and evaluated the mechanism of action of the observed cardioprotective effects of artesunate and DHA. We report here for the first time that the administration of artesunate at the onset of reperfusion attenuates the myocardial injury associated with I/R. The observed beneficial effects of artesunate are associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3β, iv) inhibition of NF-κB, and v) activation of the STAT3 (SAFE) pathway. In conclusion, as artesunate has an excellent safety profile, the above data should stimulate clinical trials in patients with acute coronary syndromes.

Key words: ischaemia; myocardial infarction; pharmacokinetics; heart disease

INTRODUCTION

Despite major advances in prevention and treatment, ischaemic heart disease remains the leading cause of morbidity in the Western world, and the number of deaths from ischaemic heart disease is also rapidly rising in the developing world. Restoration of early blood flow to the ischaemic myocardium with thrombolysis is presently the most effective therapy to limit infarct size and, thus, preserve cardiac function and geometry following myocardial infarction (MI) (1). Reperfusion alone, however, is inadequate to salvage the damaged myocardium, and may result in 'myocardial ischaemia/reperfusion (I/R) injury'. Various interventions aimed at reducing myocardial I/R injury. As the majority of these interventions are given prior to or during the ischaemic insult in animals, many of them, however, failed to be translated into clinical settings (1). Thus, there continues to be a growing need to discover novel, translatable, safe and cheap interventions that could lessen the global impact of MI on society.

In the past decade, artemisinin and its derivatives have emerged as the most effective drugs for the treatment of severe malaria. Artesunate, the only artemisinin analogue that can be administered intravenously has a very short half-life of approximately 10 min and is rapidly metabolised to dihydroartemisinin (DHA), which has a half-life of approximately 1 h and is regarded as the active metabolite of artesunate (2). In addition to its therapeutic effect in severe malaria (3), artesunate has a multitude of pharmacological properties that include anti-angiogenic as well as anti-inflammatory effects (4, 5). In this regard, the administration of artesunate upon resuscitation protects against the organ injury and dysfunction induced by severe hemorrhage and resuscitation by a mechanism that involves the activation of the Akt-endothelial nitric oxide synthase (eNOS) survival pathway, and the inhibition of glycogen synthase kinase-3β (GSK-3β) and nuclear factor kappa B (NF-κB) (6). The therapeutic effects of artesunate in myocardial I/R injury, however, are unknown.

Here we investigated the effects of artesunate and DHA in a model of transient myocardial I/R in the rat and explored the mechanism of action of the observed cardioprotective effects of artesunate and DHA. Specifically, we have investigated i) the effect of artesunate on the infarct size 2 hours after reperfusion; ii) the mechanisms underlying the observed effects of artesunate including the involvement of the RISK pathway [activation of PI3K signalling pathway (the subsequent phosphorylation Akt on Ser^{473}) and ERK 1/2 signalling pathway] and the SAFE pathway (phosphorylation of STAT3 on Tyr^{705}); iii) the involvement of downstream targets of the above pro-survival pathways; phosphorylation of GSK-3β on Ser^{9}, phosphorylation of eNOS on Ser^{1177} and activation NF-κB (measured as nuclear translocation of p65) and v) the plasma concentration-time profiles of artesunate and DHA in rats.

METHODS

Animal

The experimental protocols using animals were carried out in accordance with the approved guidelines and were approved by the Home Office, London, UK (project license: PPL 70/7348). This study was carried out on 152 male Wistar rats (Charles River Laboratories, Harlow, UK) weighing 240-340 g receiving a standard diet and water *ad libitum*. Rats were randomly assigned to sham, control and treatment groups.

Myocardial Ischaemia/Reperfusion

Myocardial ischaemia/reperfusion was carried out as described previously in this journal (7). Rats were anaesthetised with thiopentone sodium (Intraval, 120 mg/kg i.p. for induction, followed by 10 mg/kg i.v. for maintenance), a tracheostomy was performed and the animals were allowed to artificially respire using a Harvard ventilator (inspiratory oxygen concentration: 30 %; 70 strokes/min tidal volume: 8-10 mL kg^{-1}) throughout the experiment. Body temperature was maintained at 37 ± 1 ^{0}C with the aid of a rectal probe thermometer attached to a homeothermic blanket unit (Harvard Apparatus Ltd., Edenbridge, Kent. U.K.). The right carotid artery was cannulated and connected to a pressure transducer (Senso-Nor 844, Horten, Norway), to monitor mean arterial pressure and heart rate and the right jugular vein was cannulated to administer maintenance anaesthesia and drugs. A parasternal thoracotomy was performed using electrocautery followed by retraction of the thymus and resection of the pericardium. A 6-0 silk suture was placed through the myocardium at the approximate level of the left anterior descending coronary artery. A piece of polythene tubing (Smiths Medical, Watford, UK) flared at one end, was placed over the suture to form a snare occluder. The coronary artery was occluded by tightening the occluder for 25 min, then the occlusion was released to allow reperfusion for 2 h.

Quantification of myocardial tissue injury

At the end of the 2 h reperfusion period, the LAD was re-occluded and 1 ml of Evans Blue dye was administered via the jugular vein to distinguish between perfused and nonperfused (area at risk; AAR) sections of the heart. The heart was excised and immersed in ice-cold 0.9% saline to achieve cardioplegia and sectioned into 3 slices of 3-4 mm to the level of the suture. After right ventricular wall was removed, the non-perfused myocardium was separated from the perfused tissue and was weighed; AAR was expressed by a percentage of the left ventricle. Then the AAR was cut into small pieces and incubated with p-nitroblue tetrazolium (NBT, 0.5 mg/ml) for 30 min at 37 ^{0}C. NBT is reduced to a dark blue/purple azole in the presence of reducing compounds, enabling necrotic tissue to be distinguished from viable tissue. The stained tissue was separated from the infarcted tissue, weighed, and the infarct size expressed as a percentage of the AAR.

Western blot analysis

Semi-quantitative western blot analyses in heart tissues (biopsies obtained from the anterior wall – area at risk) were carried out as described previously (8-10). Heart samples were homogenised in 10 % homogenisation buffer and centrifuged at 4,000 RPM for 5 min at 4 ^{0}C, after which the supernatant was centrifuged at 14,000 RPM for 40 min to obtain the cytosolic fraction. The pelleted nuclei was resuspended in extraction buffer, incubated in ice for 30 min and centrifuged at 14,000 for 20 min at 4 ^{0}C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined on both nuclear and cytosolic extracts using a bicinchoninic acid (BCA) protein assay kit following the manufacturer's directions (Thermo Fisher Scientific, Rockford, IL). Proteins were separated by 8 % sodium dodecyl sulphate-PAGE (SDS-PAGE) and transferred to a polyvinyldenedifluoride membrane, which was then incubated with primary antibody (rabbit anti-total GSK-3β, dilution 1:200; goat anti-pGSK-3β Ser^{9} dilution 1:200, rabbit anti-total

Akt, dilution 1:1000, mouse anti-pAkt Ser^{473}, dilution 1:1000, goat anti-peNOS Ser ^{1177}, dilution 1:200, rabbit anti-eNOS, dilution 1:200; rabbit anti-NFκβ p65, dilution 1:1000; rabbit anti-total STAT3, dilution 1:1000, anti-pSTAT3 Tyr^{705}, dilution 1:1000) over night at 4 ^{0}C. Blots were incubated with secondary antibody conjugated with horseradish peroxidase (dilution 1:10,000) for 30 min in at room temperature and developed with the ECL detection system. The immunoreactive bands were visualized by autoradiography and the density of the bands was evaluated densitometrically using the Quantity One 1-D Analysis software (Bio-Rad Laboratories Ltd, Hertfordshire, UK). The membranes were stripped and incubated with β-actin monoclonal antibody for 30 min and subsequently with anti-mouse antibody for 30 min, at room temperature, in order to assess gel-loading homogeneity. Relative band intensity was assessed and normalized against parallel β-actin expression. Each group was then adjusted against corresponding sham-control data to establish relative protein expression when compared to sham-control animals.

Experimental Design

Animals were either subjected to i) surgical procedure alone with no LAD occlusion or reperfusion, and treated with vehicle (sham), or ii) surgical procedure with LAD occlusion. Animals were either treated with vehicle (DMSO 10% v/v or NaHCO3), artesunate (1 mg/kg in NaHCO3) or DHA (0.1 mg/kg in DMSO) at the start of reperfusion (bolus injection), with/without pre-treatment with inhibitors of PI3K (LY294002) or ERK (U0126) at 10 min before LAD-occlusion (0.3 mg/kg or 0.2 mg/kg, respectively). The dose of artesunate used were based on those that we have recently shown to a) reduce the organ injury/dysfunction and b) activate pro-survival pathways in anaesthetized rats subjected to severe haemorrhage and resuscitation (11). In addition, the dose of 2.4 mg/kg i.v. is the dose used in humans to treat malaria falciparum and, hence, offers the possibility for translational studies. We tried to limit the dose of artesunate used here to the maximum of 2.4 mg/kg, and (in the first instance)

tried a dose of 1 mg/kg, which already gave us a significant signal. We are currently evaluating in a single-centre phase II RCT the effects of 2.4 and 4.8 mg/kg i.v. in patients with severe haemorrhage after trauma. The doses of the pathway inhibitors used in this study were selected from the literature (12, 13). As a 'gold-standard' reference treatment animals were subjected to surgical procedure followed by either 2 cycles of ischaemic preconditioning (One cycle of IPC: 5 min ischaemia and 5 min reperfusion) with/without pre-treatment with LY294002 or U0126 10 at 10 min before LAD-occlusion.

LC-MS/MS analysis of artesunate and DHA

Blood samples (0.2 ml) were withdrawn from the cannulated carotid artery into heparinized tubes at -5, 5, 15, 30, 45, 60, 120, 180 and 240 min following the i.v. dose (injection of artesunate considered time = 0). The blood samples were centrifuged at 9900 rpm for 3 min to obtain plasma which was stored at −80°C until analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Plasma concentrations of Artesunate (AS) and Dihydroartemisinin (DHA) in samples (about 60µl) were determined by reversed phase LC-MS/MS using an adaptation of the previously described method (14). Rat plasma was purified by protein precipitation with acetonitrile, evaporation, and reconstitution in 10 mM ammonium formate /MeOH (1:1) adjusted to pH 3.9 with formic acid. Separations were done on a 2.1mm x 50mm Atlantis dC18 3 µm analytical column (Waters, Milford, MA, USA). The chromatographic system (CTC Analytics AG, Zwingen, Switzerland) was coupled to a triple stage quadrupole Thermo Quantum Discovery Max mass spectrometer equipped with an electrospray ionization interface (Thermo Fischer Scientific Inc., Waltham, MA). The selected mass transitions were *m/z* 221.1→163.1 with a collision energy (CE) of 14eV for AS and DHA, and *m/z* 226.2→168.1 (CE 20eV) for the stable isotope-labelled internal standard dihydroartemisinin-$^{13}CD_4$. Blank rat plasma pool (collected by cardiac puncture of 11 rats)

was used for the preparation of matrix-matched calibration and control samples. The calibration curves were fitted by quadratic log-log regression as factor of the peak-area ratio of DHA and artesunate to IS no IS in graphs versus the respective DHA and artesunate concentration in each standard sample. During the analysis, each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Control samples at three concentrations levels (low, medium and high: i.e. 15, 150 and 1500 ng/mL) were assayed throughout rat sample analysis. Inter-assay precision obtained with plasma QCs samples at 15, 150 and 1500 ng/mL of DHA and AS were 12.4, 12.7 and 7.4, and 13.9, 13.2 and 7.5% for DHA and AS, respectively. Mean absolute deviation from nominal values of QCs samples (15, 150 and 1500 ng/mL) during the analysis were 2.2, 5.5, 0.4 %, and 2.1, 0.1, 2.8%, for DHA and AS, respectively. The lower limit of quantification was 10 ng/ml. The Laboratory participates to the External Quality Control program for antimalarial drugs organised by the Worldwide Antimalarial Resistance Network (WWARN).

Statistics

Data is presented as mean ± standard error of the mean (SEM) of *n* observations. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The half-lives ($T_{1/2}$) of artesunate and DHA were calculated as ln2/ke, considering the slope of the decay of the elimination phase (10-20 and 10-65 min, for artesunate and DHA, respectively). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of artesunate on the infarct size in rats that underwent transient myocardial I/R injury

First, we investigated the effects of a bolus-injection upon reperfusion of artesunate (1 mg/kg; *i.v*) on the infarct size in rats that underwent transient myocardial I/R injury by LAD-occlusion. When compared to sham-animals treated with vehicle, animals subjected to LAD-occlusion and treated with vehicle demonstrated a significant increase in infarct size ($P < 0.0001$, Figure 1). The administration of artesunate, however, significantly reduced the infarct size, compared to vehicle treated animals (or $P < 0.0001$, Figure 1). To determine whether DHA, the active metabolite of many artemisinin derivatives (2), is essential for the observed cardioprotective effects of artesunate, we next investigated the effect of DHA (0.1 mg/kg; *i.v*; at the start of reperfusion) on the infarct size in rats subjected to LAD-occlusion**.** When compared to rats treated with vehicle alone, the administration of DHA significantly reduced infarct size ($P = 0.01$, Figure 1). To confirm that artesunate is, indeed, metabolised to DHA in rats with myocardial ischaemia and reperfusion, we investigated the plasma concentrations of artesunate and DHA in our experimental setting. Maximal plasma concentrations (C_{max}) of artesunate and DHA of 39.7 and 223 ng/ml respectively, were detected at a T_{max} of 10 min in sham animals (Figure 2A). Rats that underwent LAD-occlusion demonstrated a C_{max} of artesunate and DHA of 67.9 and 187.3 ng/ml respectively, at a T_{max} of 10 min (Figure 2B). The $T_{1/2}$ of artesunate and DHA were 2 and 3 min, and 13 and 10 min, in sham animals and animals subjected to LAD occlusion, respectively (Figure 2A and 2B).

Effect of artesunate on the phosphorylation of Akt and GSK-3β, in the hearts of rats that underwent transient myocardial I/R injury

We have previously reported that artesunate activates Akt, inhibits GSK-3β and inhibits the activation of NF-κB in liver and kidney of rats subjected to severe haemorrhage (which leads to organ ischaemia) and resuscitation (which leads to reperfusion). In order to elucidate whether the same survival pathways are activated by artesunate in the heart, we have investigated the effects of artesunate on the degree of phosphorylation of Akt on Ser^{473} and on GSK-3β on Ser^{9}. (15). When compared to sham animals, rats subjected to LAD-occlusion and treated with vehicle demonstrated no changes in the phosphorylation of Akt on Ser^{473} or GSK-3β on Ser^{9} (both $P > 0.5$, Figures 3A, 3B). The administration of artesunate, however, resulted in a significant increase in the phosphorylation of both Akt on Ser^{473} and GSK-3β on Ser^{9} ($P = 0.005$ and $P = 0.003$, respectively, Figures 3A, 3B), further indicating that the observed cardioprotective effects of artesunate are associated with the activation (phosphorylation) of Akt.

Effect of artesunate on the nuclear translocation of the p65 NF-κB subunit in the heart of rats that underwent transient myocardial I/R injury

When compared with sham animals, rats subjected to LAD-occlusion exhibited a significant increase in the nuclear translocation of the p65 subunit ($P = 0.008$, Figure 3C) indicating an activation of NF-κB. The administration of artesunate, however, significantly attenuated the increase in nuclear translocation of p65 and, hence, resulted in an inhibition of the activation of NF-κB in rats subjected to LAD-occlusion ($P = 0.02$, Figure 3C).

Effect of artesunate on the expression of eNOS in the hearts of rats that underwent transient myocardial I/R injury

As activation of the endothelial nitric oxide synthase (eNOS) has anti-apoptotic, anti-inflammatory, and antioxidant effects, and is hence is cardioprotective (16), we next investigated the effects of artesunate on the degree of phosphorylation of eNOS on Ser^{133}. When compared to sham animals, LAD-occlusion did not affect the phosphorylation of eNOS on Ser^{1177} ($P > 0.5$, Figure 3D). The administration of artesunate, however, resulted in a significant increase in the phosphorylation of eNOS on Ser^{1177} in rats subjected to LAD occlusion and reperfusion ($P = 0.003$, Figure 3D).

Effect of artesunate on the phosphorylation of STAT3, in the heart of rats that underwent transient myocardial I/R injury

As STAT3 is a vital mediator in the SAFE pathway and is known to mediate cardioprotection when activated by mechanical procedures (e.g. pre- and postconditioning) (17) (18), we next investigated the effects of artesunate on the degree of phosphorylation of STAT3 on Tyr^{705}. When compared to sham animals, rats subjected to LAD-occlusion demonstrated no change in the phosphorylation of STAT3 on Tyr^{705} ($P > 0.05$, Figures 3E). The administration of artesunate, however, resulted in a significant increase in the phosphorylation of STAT3 on Tyr^{705} ($P = 0.03$, Figures 3E).

Effect of artesunate on the infarct size, in animals that underwent transient myocardial I/R injury following inhibition of survival pathways

The above data provide some evidence that artesunate activates key elements of the RISK pathways after LAD-occlusion and reperfusion. It is not clear from the above data whether inhibition of key elements of the RISK pathway also attenuates the cardioprotective effects of artesunate (cause-effect relationship). Thus, we investigated the roles of PI3K/Akt and ERK 1/2 (e.g. the signalling events know to activate the RISK survival pathway (12, 13) in the cardioprotective effects of artesunate. Therefore, rats were pre-treated (10 min prior to

ischaemia) with inhibitors of either PI3K (LY294002, 0.3 mg/kg; *i.v*) (12) or ERK 1/2 (U0126, 0.2 mg/kg; *i.v*) (13). When compared to LAD-subjected animals treated with artesunate and no prior inhibition of either PI3K or ERK, the administration of either LY294002 or U0126 significantly abolished the reduction of the infarct size by artesunate, indicating that the activation by artesunate of both the PI3K/Akt and the ERK 1/2 pathways are essential for the cardioprotective effects of artesunate ($P < 0.0001$, Figure 4).

Effect of inhibition of the RISK pathway on infarct size, in rats that underwent transient myocardial I/R injury subsequent to ischaemic preconditioning (IPC)

Having shown that activation by artesunate of both the PI3K/Akt and the ERK 1/2 pathways are essential for the cardioprotective effects of artesunate, we speculated that artesunate protects the heart through mechanisms that are similar to those of ischaemic preconditioning (IPC). In order to confirm that activation of the RISK pathway is pivotal in mediating the IPC-induced cardioprotection, rats were treated with LY294002 or U0126 at 10 min prior to initiating the IPC stimulus. When compared to animals subjected to LAD-occlusion and treated with vehicle, 2 cycles of IPC before occlusion of the LAD resulted in a significant reduction in infarct size ($P < 0.0001$, Figure 5). Like the cardioprotective effect of artesunate, the cardioprotective effect afforded by IPC was also abolished by LY294002 and U0126 ($P = 0.0004$ and $P = 0.002$, respectively, Figure 5), indicating that the activation of both the PI3K/Akt and the ERK 1/2 pathways are essential for the cardioprotective effects of both artesunate and IPC.

DISCUSSION

We report here for the first time that a single *i.v.* injection upon reperfusion of the safe, antimalarial drug artesunate reduces myocardial infarct size in the rat *in vivo*. Notably, the observed beneficial effects of artesunate were associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3β, iv) inhibition of NF-κB, and v) activation of the STAT3 (SAFE) pathway, while inhibitors of the RISK pathway (Both PI3K and ERK) abolished the cardioprotective effects of artesunate.

Activation of the PI3K/Akt/eNOS survival pathway is known to protect the myocardium against I/R injury (19). Indeed, we report here that the cardioprotective effects afforded by artesunate were a) associated with phosphorylation of Akt on Ser^{473}, and b) abolished by the specific PI3K-inhibitor LY294002, suggesting that the artesunate-induced cardioprotection is secondary to the activation of the PI3K/Akt-survival pathway. Akt phosphorylates eNOS and the resulting eNOS activation has anti-apoptotic, anti-inflammatory and antioxidant effects. The administration of artesunate was associated with an increase in the phosphorylation of eNOS on Ser^{1177}, secondary to activation of the PI3K/Akt pathway. Moreover, we report here that the cardioprotective effects of artesunate were associated with Ser^{9} phosphorylation and, hence, inactivation of GSK-3β in the heart, both of which are known to modulate various cellular processes including survival and apoptosis. Moreover, inhibition of GSK-3β prevents the nuclear translocation of p65 and, hence the activation of NF-κB (20). The observed cardioprotective effects of artesunate were associated with both Ser^{9} phosphorylation of GSK-3β (see above) and reduction of the nuclear translocation of p65 from the cytosol to the nucleus (in the ischaemic heart). Recently, Collino and colleagues demonstrated that inhibition of GSK-3β with either insulin or TDZD-8 was associated with a significant reduction of the nuclear NF-κB activity, in diabetic rats subjected to cereberal I/R injury (21).

Blockade of NF-κB activation by NF-κB decoys (22), or a vitamin E-like antioxidant (23) has been reported to attenuate myocardial I/R injury and to improve functional recovery of the heart after an ischaemic event. Thus, it is likely that the observed effects of artesunate are (at least in part) attributable to the reduced activation of NF-κB.

Members of the MAPK cascade have been implicated in regulating myocyte survival after I/R injury and oxidative stress. ERK 1/2 signalling has been identified as one of the major components of the RISK pathway. We report here that the ERK 1/2 inhibitor, U0126, abolished the cardioprotective effects afforded by artesunate, indicating that the activation of ERK 1/2 importantly contributes to the cardioprotective effects of artesunate. Indeed, the activation of the ERK pathway during reperfusion leads to cardioprotection (24, 25).

Additionally, activation of the SAFE pathway may mediate the cardioprotective effects of ischaemic pre- and postconditioning. STAT3 is a latent transcription factor, plays a role in various cellular functions including proliferation, differentiation and cell survival (26). We demonstrate here that the cardiprotective effects of artesunate in the rat heart were also associated with activation of STAT3. There is good evidence that activation of STAT3 in the heart confers cardioprotection as the myocardial infarct size caused by I/R in mice with cardiac-specific expression of constitutively active STAT3 is significantly smaller than the infarct size caused by I/R results in non-transgenic littermates (27). Our results clearly show that artesunate caused activation of both the RISK and the SAFE pathways. Thus, our study confirm previous data which have identified a role for SAFE signaling in the initiation of the activation of the RISK pathway, which plays a pivotal role in the cardioprotection afforded by pre- and post-conditioning (28, 29). Whether enhanced activation of SAFE further promotes RISK activation or vice versa cannot be distinguished in our study. However, our

data suggest that targeting a single pathway as a sole therapeutic target may not be sufficient (or at least less effective) than targeting both pathways. Interestingly, we also report that artesunate causes both STAT3 activation and NF-κB inhibition. Inhibition of NF-κB activation secondary to STAT3 activation inhibits NF-κB-mediated hypertrophic and inflammatory gene expression by blocking NF-κB activation in the heart (30). However, exactly how this occurs and the temporal pattern of the reported cross-talk between STAT3 NF-κB are still unclear.

In vivo, artesunate is rapidly hydrolysed to DHA, which is the common active metabolite that mediates the beneficial effects of many artemisinin derivatives in patients with malaria (31). The conversion from artesunate to DHA occurs rapidly following *i.v.* administration, indicated by the swift decline in artesunate concentrations in the early post-dose period (32). DHA metabolism occurs through conjugation by the UDP-glucuronosyltransferase system, with UGT1A9 and UGT2B7 being the main responsible isoforms (33). The metabolism of DHA occurs more slowly, with an average half-life of DHA of 1 – 2 h after bolus injection of artesunate (32). In our study, peak plasma concentrations of both artesunate (39.7 ng/ml) and DHA (223 ng/ml) were achieved in the rat within 10 min of the injection of artesunate. This demonstrates a very rapid conversion of artesunate to DHA in the rat, and this metabolism was unaltered in rats subjected to myocardial I/R. The peak plasma concentrations seen here in the sham treated rats are much lower than those exhibited in healthy human volunteers, where a similar dose of artesunate (1 mg/kg) resulted in a peak plasma concentration of artesunate and DHA of around 6128 ng/ml and 800 ng/ml, respectively (34). The efficacy of artesunate in malaria has been attributed to i) attaining a high rapid initial C_{max}, and ii) its swift hydrolysis to DHA (35). This could mean that DHA is more potent than artesunate in reducing parasite load in malaria, but also indicates that DHA may mediate the

cardioprotective effects of artesunate. We demonstrate here that a dose of 0.1 mg/kg of DHA caused a significant reduction in infarct size, while the same dose of artesunate was not effective (data not shown). This indicates that DHA is the effective mediator of the cardioprotective effects of artesunate (although we cannot exclude that artesunate has at least some cardioprotective effects in its own right).

Limitations of the study: We report that the i.v. injection of artesunate upon reperfusion reduces infarct size when measured in anaesthetized, ventilated rats after a relatively short (2 hours) period of reperfusion. Further studies are warranted that confirm that artesunate reduces the size of necrosis and improves contractility (function) after longer periods of reperfusion (ideally 1-2 weeks). We are unable to provide definite prove that DHA is, indeed, the active metabolite, which mediates the cardioprotective effects of artesunate, as we are unable to block the metabolism of artesunate to DHA. We cannot exclude that the pathway inhibitors used in this study have 'unknown', non-specific effects, which may have contributed to 'prevention' of the cardioprotective effects of artesunate documented in this study. This could, of course, be true for any pharmacological intervention used during an in vivo experiment and, hence, is not a specific limitation of this study.

In conclusion, we report here for the first time that the administration of low doses (1 mg/kg i.v) artesunate at the onset of reperfusion attenuates the myocardial injury associated with I/R. The observed beneficial effects of artesunate are associated with i) activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, ii) activation of eNOS, iii) inhibition of GSK-3β, iv) inhibition of NF-κB, and v) activation of the STAT3 (SAFE) pathway. Most notably, the cardioprotective effects of artesunate were abolished by inhibition of PI3K and ERK1/2 indicating that activation by artesunate of these pathways is pivotal for the cardioprotective

effects of artesunate. Artesunate is rapidly metabolised to DHA *in vivo* and this metabolism is not altered in animals with myocardial infarction. DHA also reduces myocardial infarct size in the rat indicating that DHA may be the active metabolite of artesunate. As the administration of higher doses of artesunate is safe in healthy volunteers and patients with severe malaria, artesunate may represent a novel highly translatable approach for the therapy of acute MI in man.

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AUTHOR CONTRIBUTIONS

CT and AIK designed research; AIK, AK, JC, MR, TM, LD and MC performed research; AIK, AK, JC, LM, MR, TM, LD, and MC analysed data; and AIK, JC, LM and CT wrote the paper.

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FIGURES LEGENDS

Figure 1. Effect of artesunate or dihydroartemisinin on infarct size in rats that underwent transient myocardial I/R injury. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD), and treated with vehicle (sham, *n* = 6), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion and treated with either vehicle (*n* = 10), artesunate (ARS; 1 mg/kg; *n* = 10) or dihydroartemisinin (DHA; 0.1 mg/kg, *n* = 6) at the start of reperfusion. AAR, area at risk. Data are expressed as means ± SEM for *n* number of observations. *$P < 0.0001$ *vs. MI + vehicle;* §$P < 0.0001$ *vs. MI + ARS.*

Figure 2. Plasma concentration-time profiles of artesunate and dihydroartemisinin following a single intravenous dose of artesunate. Rats were either subjected to (left) surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD) or (right) surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion. Rats were treated with artesunate (ARS; 1 mg/kg) at the start of reperfusion. Plasma concentrations of artesunate and dihydroartemisinin (DHA) were measured in blood samples withdrawn from the cannulated carotid artery at -5, 5, 15, 30, 45, 60, 120, 180 and 240 min following bolus-injection of artesunate. Data are expressed as means ± SEM for four numbers of observations.

Figure 3. Effect of artesunate on signalling pathways in the heart of rats that underwent transient myocardial I/R injury. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD), and treated with vehicle (sham), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion and treated with either vehicle or artesunate (ARS; 1 mg/kg) at the start of

reperfusion. Heart samples were collected at the end of the 120 min reperfusion period. Densitometric analysis of the bands is expressed as relative optical density (O.D) of (A) Akt phosphorylation ($pSer^{473}$) corrected for the corresponding total Akt content; (B) glycogen synthase kinase (GSK)-3β phosphorylation ($pSer^{9}$) corrected for the corresponding total GSK-3β content; (C) NF-κB p65 subunit levels in both cytosol and nuclear fractions expressed as a nucleus/cytosol ratio; (D) endothelial nitric oxide synthase (eNOS) phosphorylation ($pSer^{1177}$) corrected for the corresponding total eNOS content; (E) signal transducer and activator of transcription 3 (STAT3) phosphorylation at Tyr^{705} ($pSTAT3^{705}$), corrected for the corresponding total STAT3 content. All values were normalized using the related sham-operated band. Data is expressed as means ± SEM for four observations. *$P < 0.0001$ *vs. MI + vehicle.*

Figure 4. Effect of inhibition of components of the RISK pathway on infarct size in rats treated with artesunate that underwent transient myocardial I/R injury. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior descending coronary artery (LAD), and treated with vehicle (sham, $n = 6$), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion and treated with either vehicle ($n = 10$) or artesunate (ARS; 1 mg/kg; $n = 10$) at the start of reperfusion with or without pre-treatment with LY29400 (LY; 0.3 mg/kg; $n = 10$) or U0126 (0.2 mg/kg; $n = 10$) at 10 min before LAD-occlusion. AAR, area at risk. Data are expressed as means ± SEM for *n* number of observations. *$P < 0.0001$ *vs. MI + vehicle;* §$P < 0.0001$ *vs. MI + ARS.*

Figure 5. Effect of inhibition of components of the RISK pathway on infarct size in rats that underwent transient myocardial I/R injury subsequent to an IPC stimulus. Rats were either subjected to surgical procedure alone with no occlusion of the left anterior

descending coronary artery (LAD), and treated with vehicle (sham, *n* = 6), or surgical procedure with LAD-occlusion (MI) for 25 min and 120 min reperfusion. For ischaemic preconditioning, 2 cycles of ischaemic preconditioning (IPC; 1 cycle: 5 min ischaemia and 5 min reperfusion) have been performed with or without pre-treatment with LY29400 (LY; 0.3 mg/kg; *n* = 6) or U0126 (0.2 mg/kg; *n* = 6) at 10 min before LAD-occlusion. AAR, area at risk. Data are expressed as means ± SEM for *n* number of observations. **P < 0.0001 vs. MI + vehicle;* §*P < 0.0001 vs. MI + IPC + Vehicle.*

Figure 1

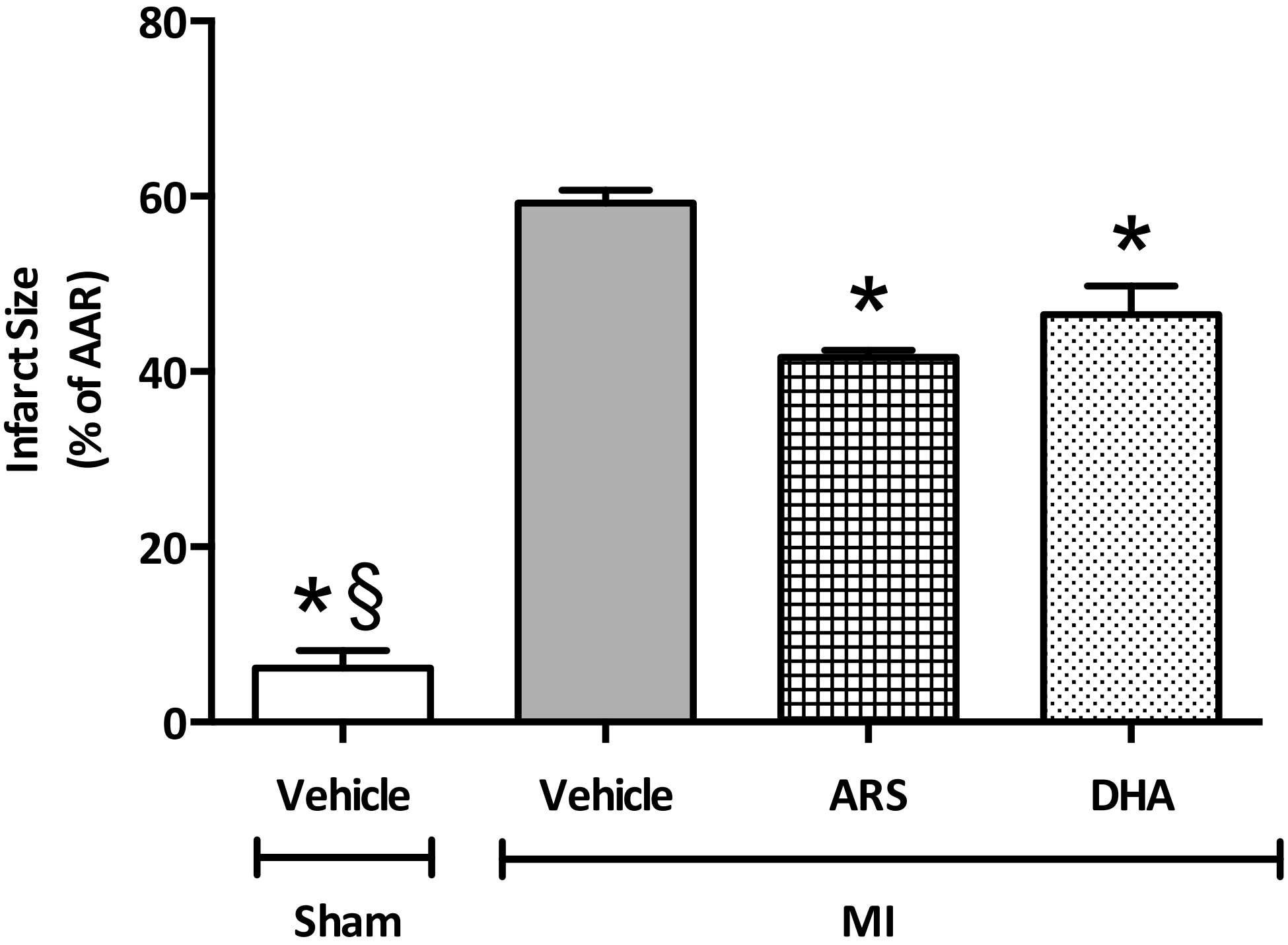
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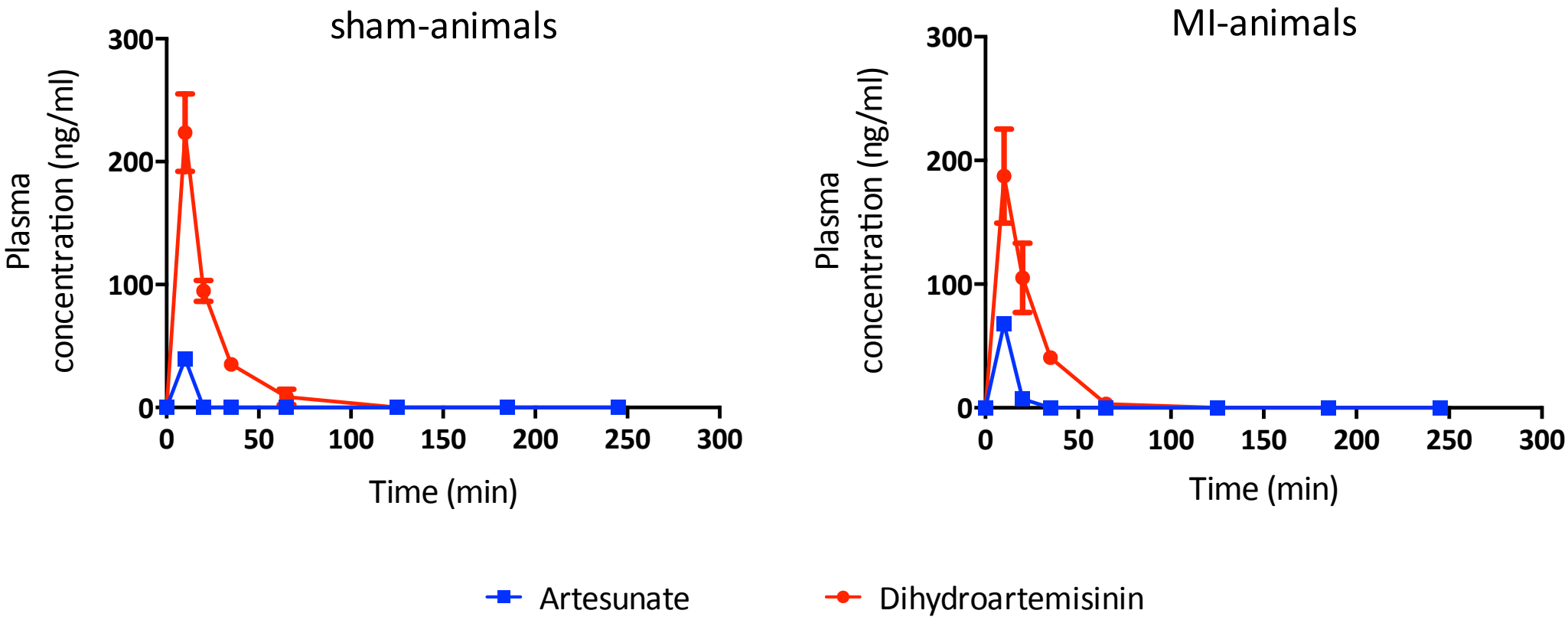
Figure 2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250
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Time (min)
Artesunate
Dihydroartemisinin

Figure 3

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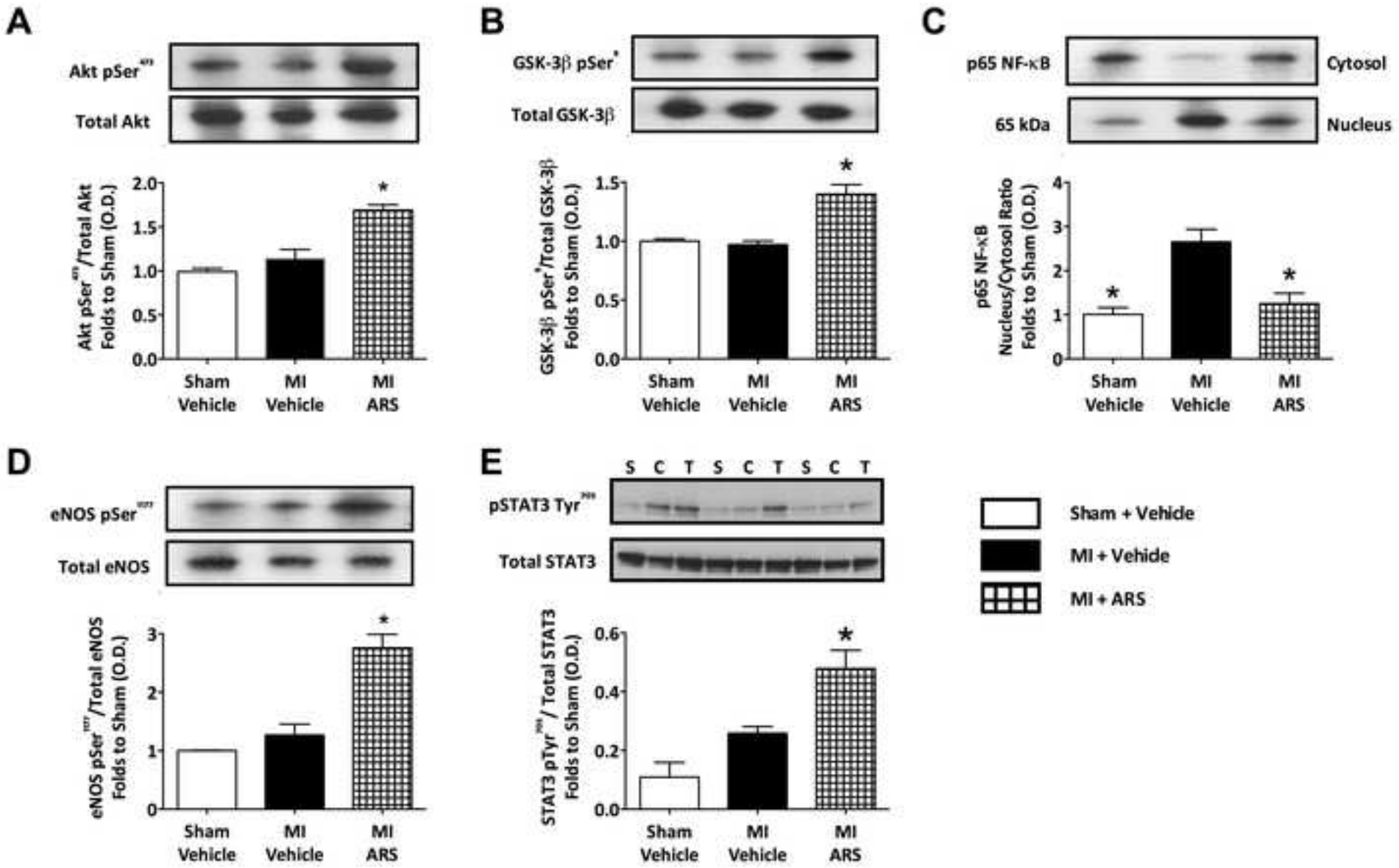

Figure 4

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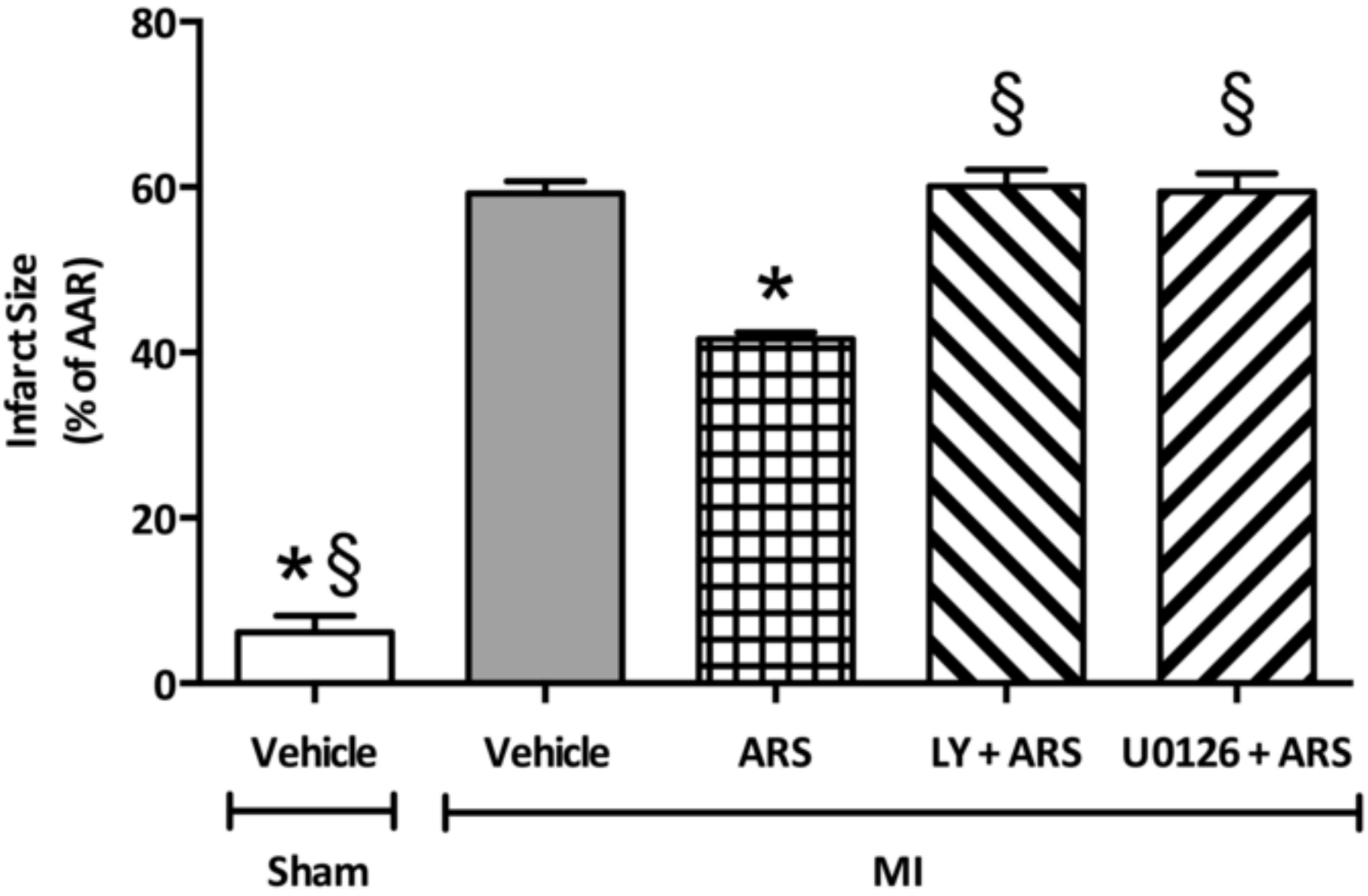
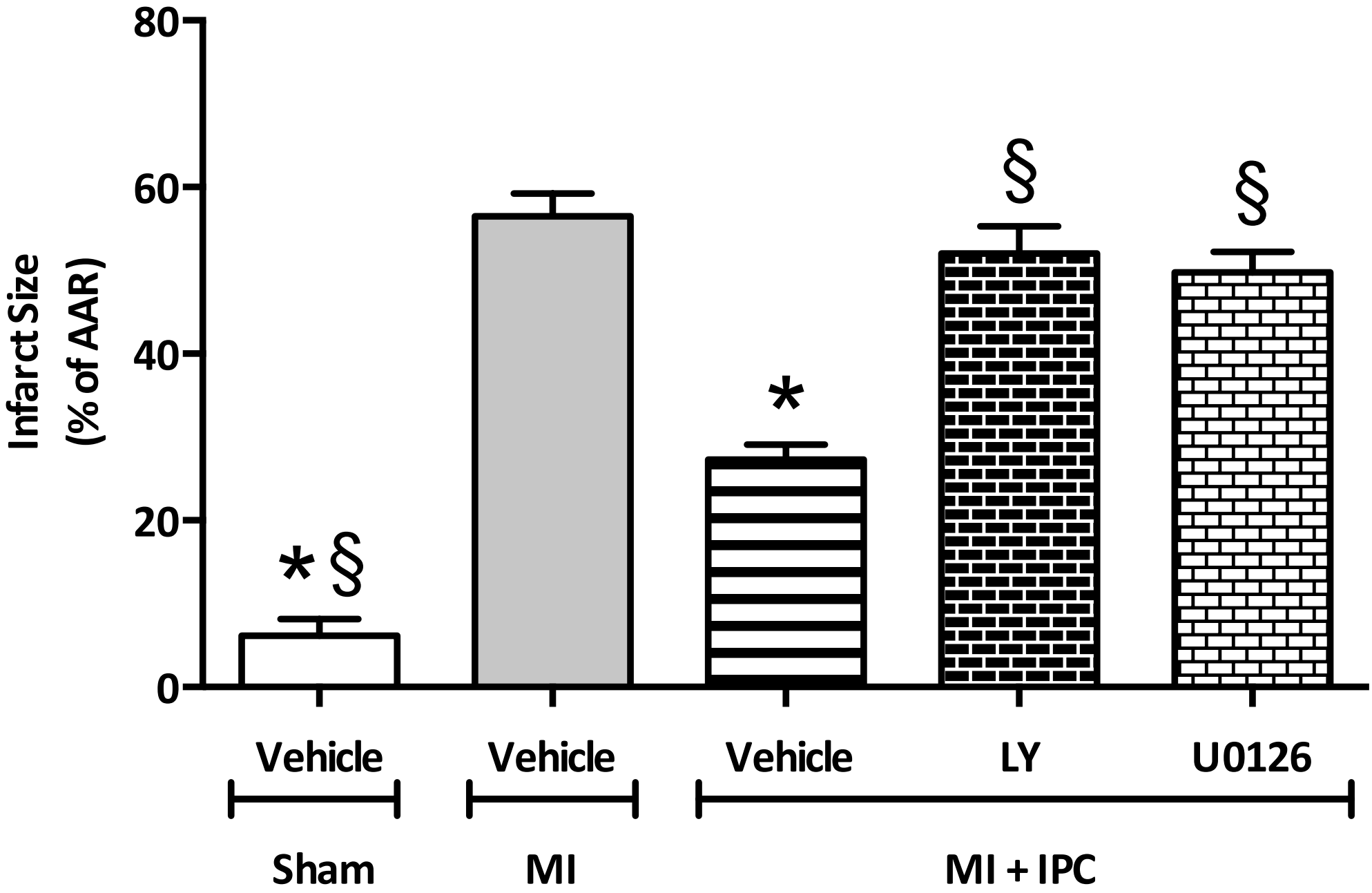


Figure 5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